Miltenyi Biotec

T Cell Activation/ Expansion Kit
non-human primate

Order no. 130-092-919

Contents

1. Description

Components	**2 mL Anti-Biotin MACSiBead™ Particles, cell culture grade**, corresponding to 4×10^8 MACSiBead Particles; MACSiBead Particles conjugated to monoclonal anti-biotin antibodies, **0.4 mL CD2-Biotin, human** (100 µg/mL), **0.4 mL CD3-Biotin, non-human primate** (100 µg/mL), **0.4 mL CD28-Biotin, human** (100 µg/mL).
Product format	All components are supplied in azide-free buffer containing stabilizer. Low endotoxin.
Storage	Store protected from light at 2 – 8 °C. Do not freeze. The expiration date is indicated on the vial label.

This product is applicable for the activation and expansion of T cells from rhesus monkey (*Macaca mulatta*). The CD3 antibody provided with the kit has also been tested to react with cynomolgus monkey (*M. fascicularis*) and pigtail monkey (*M. nemestrina*). The CD2 and CD28 antibodies provided with the kit have also been tested to cross-react with cynomolgus monkey (*M. fascicularis*). Cross-reactivity with other non-human primates has not been tested.

1.1 Principle of the T Cell Activation/Expansion Kit

The T Cell Activation/Expansion Kit is designed to activate and, if required, expand rhesus monkey T cells. The kit consists of Anti-Biotin MACSiBead Particles and biotinylated antibodies against non-human primate CD3 and human CD2 and CD28. Anti-Biotin MACSiBead Particles loaded with biotinylated antibodies are used to mimic antigen-presenting cells and activate resting T cells from peripheral blood mononuclear cells (PBMCs) as well as purified T cells. T cell expansion is achieved by culturing and reactivation at day 14 of culture.

1.2 Background information

The Anti-Biotin MACSiBead Particles are in a first step loaded with biotinylated antibodies. An optimal activation is achieved by using equal amounts of the provided biotinylated antibodies against CD2, CD3, and CD28.

▲ **Note:** Other combinations of biotinylated antibodies may be experimentally tested for their suitability, if required.

Loaded Anti-Biotin MACSiBead Particles are subsequently used for the activation of T cells. An optimal activation of T cells is accomplished by using one loaded Anti-Biotin MACSiBead Particle per two cells (bead-to-cell ratio 1:2). The cells are cultured for up to 3 days and can be further expanded, if desired. Expansion is achieved by adding IL-2 and fresh medium every 2–3 days. Cells are restimulated at day 14 by adding additional loaded Anti-Biotin MACSiBead Particles at a bead-to-cell ratio of 1:2.

▲ The efficiency of the T cell activation depends on their differentiation status, which will often be heterogenous. For special applications, it is therefore recommended to experimentally determine the best stimulation ratio.

▲ Over-activation of T cells carries a risk of activation-induced cell death.

T cells, activated by using Anti-Biotin MACSiBead Particles, can be used for any downstream processing such as cytokine analysis or immunoprecipitation. Also, activated T cells can be transfected with high efficiency.

Anti-Biotin MACSiBead Particles show no autofluorescence and do not need to be removed prior to flow cytometric analysis. However, if desired, removal of Anti-Biotin MACSiBead Particles is easily achieved by using the MACSiMAG™ Separator (see 2.6).

1.3 Applications

- Activation and expansion of resting T cells from rhesus monkey PBMCs.
- Expansion of antigen-specific T cells.

Miltenyi Biotec B.V. & Co. KG
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macsde@miltenyi.com
www.miltenyibiotec.com

1.4 Reagent and instrument requirements

- Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS® BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.

  ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as non-human serum albumin, non-human serum, or fetal bovine serum (FBS). Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- Medium X-VIVO 15™ (Cambrex) supplemented with 10% FCS.

  ▲ **Note:** 2-Mercaptoethanol (0.01 mM) can be added to preserve cell viablity in case of rapid cell growth.

- (Optional) T Cell Expansion Bags or flat bottom cell culture plates with lids.
- Humidified incubator.
- MACSmix™ Tube Rotator (# 130-090-753) for loading of MACSiBead Particles.
- (Optional) MACSiMAG™ Separator for removal of Anti-Biotin MACSiBead Particles after T cell expansion prior to downstream experiments.

  ▲ **Note**: Do not remove MACSiBead Particles by using MACS Columns and MiniMACS™, MidiMACS™, SuperMACS™, or autoMACS Pro Separator.

- (Optional) Respective flurochrome-conjugated REAfinity™ Antibodies for flow cytometric analysis, e.g., CD4 Antibody, CD8 Antibody, CD25 Antibody, CD69 Antibody. For more information about fluorochrome-conjugated antibodies see www.miltenyibiotec.com.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD Staining Solution (# 130-111-568) for the flow cytometric exclusion of dead cells.
- (Optional) Respective MACS Cytokines, e.g., Human IL-2 IS.

2. Protocol

▲ All steps in the protocol have to be performed under sterile conditions.

2.1 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™.

▲ **Note:** To remove platelets after density gradient separation, resuspend cell pellet in buffer and centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

When working with tissues or lysed blood, prepare a single-cell suspension using standard methods.

For details see the protocols section at www.miltenyibiotec.com/protocols.

▲ Dead cells may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).

2.2 Loading of Anti-Biotin MACSiBead™ Particles

▲ Resuspend Anti-Biotin MACSiBead Particles thoroughly by vortexing before use, to obtain a homogenous suspension.

▲ Anti-Biotin MACSiBead Particles are supplied without preservative. Remove aliquots under aseptic conditions.

▲ It is recommended to load Anti-Biotin MACSiBead Particles in batches of 1×10^8 Anti-Biotin MACSiBead Particles. Loaded Anti-Biotin MACSiBead Particles are stable for up to 4 months when stored at 2–8 °C.

1. Pipette 100 µL of CD2-Biotin, 100 µL of CD3-Biotin, and 100 µL of CD28-Biotin into a sealable 2 mL tube and mix well.

   ▲ **Note:** This antibody combination, with a final antibody concentration of 10 µg per antibody per 1 mL loaded Anti-Biotin MACSiBead Particles, is optimized for achieving maximal T cell activation.

2. Resuspend Anti-Biotin MACSiBead Particles thoroughly by vortexing.
3. Remove 500 µL of Anti-Biotin MACSiBead Particles (1×10^8 Anti-Biotin MACSiBead Particles) and add to antibody mix.
4. Add 200 µL of buffer to adjust to a total volume of 1 mL.

   ▲ **Note:** Anti-Biotin MACSiBead Particles can be loaded in a flexible manner with biotinylated antibodies or ligands other than those supplied. If desired, add other biotinylated antibodies or ligands at appropriate concentrations, and adjust with buffer to a total volume of 1 mL accordingly.

5. Incubate for 2 hours at 2–8 °C under constant, gentle rotation by using the MACSmix Tube Rotator (# 130-090-753) at approximately 4 rpm (slowest permanent run program).
6. The **loaded Anti-Biotin MACSiBead Particles** (1×10^8 Anti-Biotin MACSiBead Particles/mL) are now ready to use. **Do not remove the loaded Anti-Biotin MACSiBead Particles from the antibody mix.** Store at 2–8 °C for up to 4 months.

2.3 T cell activation protocol

This T cell activation protocol is optimized for strong activation of PBMCs, using one loaded Anti-Biotin MACSiBead Particle per two PBMCs or T cells (bead-to-cell ratio 1:2). The same bead-to-cell ratio is recommended when activating purified T cells.

▲ **Note:** Other ratios than 1:2 of loaded Anti-Biotin MACSiBead Particles per cell may be required for other applications (see also 1.2).

▲ Volumes for activation given below are for 5×10^6 PBMCs. When working with higher cell numbers, scale up all reagent volumes and total volumes, accordingly, e.g., for 1×10^7 total cells, use twice the volume of all indicated reagent volumes and total volumes.

▲ For the activation of purified T cells, cultivate T cells at 2.5×10^6 cells per mL per cm^2.

1. Resuspend **loaded Anti-Biotin MACSiBead Particles** thoroughly and transfer 25 µL (2.5×10^6 loaded Anti-Biotin MACSiBead Particles) per 5×10^6 PBMCs to a suitable tube.

   ▲ **Note:** If unloaded MACSiBead Particles shall be used for negative control experiments, replace loaded Anti-Biotin MACSiBead Particles by adding 12.5 µL (2.5×10^6 beads) of unloaded Anti-Biotin MACSiBead Particles per 5×10^6 PBMCs.

2. Add 100–200 µL of culture medium to the **loaded Anti-Biotin MACSiBead Particles** and centrifuge at 300×g for 5 minutes.
3. Aspirate supernatant completely and resuspend **loaded Anti-Biotin MACSiBead Particles** in 100 µL of fresh culture medium.

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4. Resuspend PBMCs at a density of 5×10^6 cells per 900 µL of culture medium X-VIVO 15™ supplemented with 10% FCS.
5. Add the prepared Anti-Biotin MACSiBead Particles from step 3 to the 900 µL of cell suspension and mix well.
6. Add the mixture to a suitable cell culture vessel at a density of 5×10^6 cells per mL per cm^2.
7. Incubate at 37 °C, 5–10% CO_2 for up to 3 days.
   ▲ **Note:** Inspect cultures daily, and add fresh medium if required.
8. For T cell expansion, proceed to step 2 of the T cell expansion protocol (see section 2.4). For immunofluorescent staining proceed to section 2.5.

2.4 T cell expansion protocol

As T cells are activated and expand, the addition of further growth factors and stimuli, such as more loaded Anti-Biotin MACSiBead Particles, is required. The following procedure is a guideline for the stimulation and expansion of T cells.

▲ Inspect cell culture daily. Depending on the expansion rate, it might be necessary to split culture every 2–3 days.

1. Perform steps 1–7 as described under section 2.3 (T cell activation protocol).
2. At day 3 gently pipette culture up and down to break up clumps.
3. Count T cells and dilute cells to $1–2\times10^6$ T cells per mL by adding culture medium supplemented with 100 units rIL-2 per mL. Incubate at 37 °C, 5–10% CO_2.
   ▲ **Note:** If rIL-2 interferes with downstream experiments, it may be omitted. However, omission will lower the cell viability.
4. Daily inspect cell culture and cultivate cells until day 14.
   ▲ **Note:** If necessary, split culture and add fresh medium supplemented with 100 units rIL-2 per mL.
5. At day 14, resuspend cells at 2.5×10^6 cells per mL in fresh medium supplemented with 100 units rIL-2 per mL.
6. For longer expansion, cells are now restimulated by adding one **loaded Anti-Biotin MACSiBead Particle** per two cells: Count cells and add 12.5 µL of **loaded Anti-Biotin MACSiBead Particles** per 2.5×10^6 T cells.
7. Further cultivate cells and repeat steps 2 and 3 every 2–3 days.
8. Proceed to downstream application, e.g., analysis of cells.
   ▲ **Note:** Removal of Anti-Biotin MACSiBead Particles is not required for immunofluorescent staining. For assays where T cells are required to return to a fully resting state before further stimulus, Anti-Biotin MACSiBead Particles should be removed at least 24 hours before restimulation (see 2.6).

2.5 Immunofluorescent staining

▲ Volumes for fluorescent labeling given below are for 10^6 total cells. When working with fewer than 10^6 cells and up to 10^7 cells, use the same volumes as indicated.

▲ MACSiBead Particles show no autofluorescence and do not need to be removed prior to flow cytometric analysis.

▲ After short term stimulus for up to 24 h, scatter properties of cells may be altered due to strong interaction between cells and MACSiBead Particles.

▲ Upon stimulation, expression of CD3 might be transiently down-regulated. Thus, the staining of CD3 on the cell surface of activated cells might be affected.

1. Resuspend cells to break up cell clumps.
   ▲ **Note:** In short-term stimulus of cells (up to 24 hours), the Anti-Biotin MACSiBead Particles may be bound strongly to cells. Care should be taken to thoroughly resuspend cells before analysis.
2. Wash, e.g. 10^6, cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cells with buffer and add staining antibodies according to the manufacturer's recommendations.
4. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
   ▲ **Note:** Working on ice requires increased incubation time. Higher temperatures and/or longer incubation times lead to non-specific cell labeling.
5. Wash cells by adding 1–2 mL of buffer per 10^6 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy.

2.6 Removal of Anti-Biotin MACSiBead Particles

▲ Removal of MACSiBead Particles may be required before magnetic separation of cells with MACS MicroBeads or before restimulation with different agents or antigens.

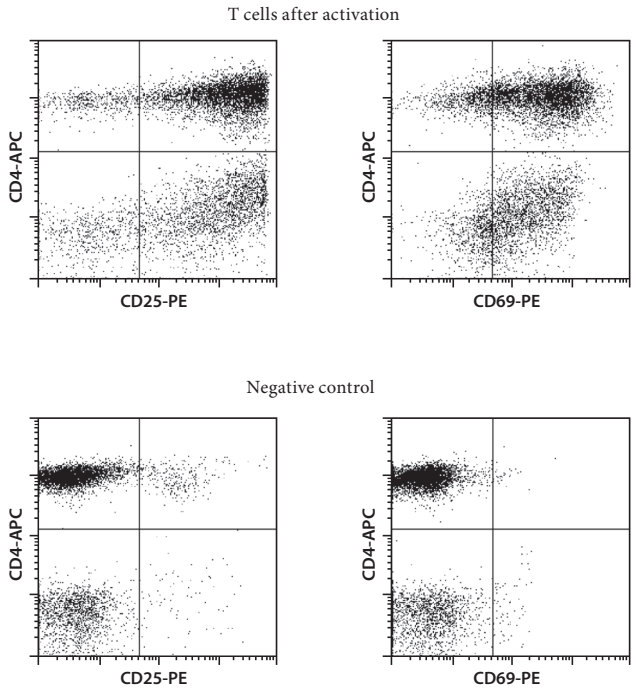
1. Harvest cells and transfer to a 5 mL, 15 mL, or 50 mL tube and wash once with buffer.
2. Resuspend cells in buffer at a density of up to 2×10^7 cells per 1 mL and vortex thoroughly.
3. Place the tube in the magnetic field of the MACSiMAG Separator.
   ▲ **Note:** Use tube rack to insert 5 mL tube into the magnetic field of the separator. For details, see MACSiMAG Separator data sheet.
4. Allow the MACSiBead Particles to adhere to the wall of the tube:

5 mL tubes:	2 minutes
15 mL or 50 mL tubes:	4 minutes

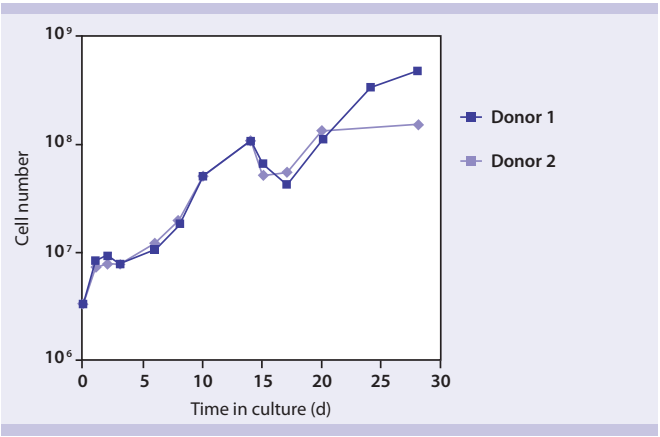
5. Retaining the tube in the magnet, carefully remove the supernatant containing the MACSiBead-depleted cells and place in a new tube.
6. Remove the tube from the separator and add buffer to the same volume as before.
7. Vortex sample, replace tube in the MACSiMAG Separator and repeat steps 4–5.
8. Collected cells can now be further processed as required.

3. Example of T cell activation and T cell expansion using the T Cell Activation/Expansion Kit

A) Anti-Biotin MACSiBead Particles were loaded with biotinylated CD2, CD3, and CD28 antibodies. Rhesus monkey PBMCs were activated using 1 loaded Anti-Biotin MACSiBead Particle per 2 cells for 72 hours. The negative control experiment was performed without adding MACSiBead Particles. Cells were fluorescently stained using CD4-APC and CD25-PE, or CD4-APC and CD69-PE, and CD8-FITC, and analyzed by flow cytometry. Cells were gated on $CD4^+$ and $CD8^+$ T cells. Dead cells and debris were excluded from the analysis based on scatter signals and propidium iodide (PI) fluorescence.



B) Anti-Biotin MACSiBead Particles were loaded with biotinylated CD2, CD3, and CD28 antibodies. Rhesus monkey PBMCs were stimulated using 1 loaded Anti-Biotin MACSiBead Particle per 2 cells for 72 hours. Cells were expanded in X-VIVO 15™ supplemented with 10% FCS and 100 U rIL-2/mL. At day 14, additional loaded Anti-Biotin MACSiBead Particles were added at a bead-to-cell ratio of 1:2. Cells were further expanded for 2 weeks (donor 1: Indian rhesus monkey, donor 2: Chinese rhesus monkey).



4. Appendix: Flask and dish sizes for T cell expansion

For T cell expansion the cells should be resuspended in culture medium at 2.5×10^6 cells/mL. The cells should be plated at a density of 2.5×10^6 cells/cm^2. Both the dilution and the cell density are important to assure optimal stimulation and cell growth.

The following table lists culture plate, dish and flask sizes suitable for different cell numbers. It also indicates the appropriate amount of medium to add.

Total cell number	Medium volume to add	Culture plate	Well diameter
0.75×10^6	0.3 mL	96 well	0.64 cm
2.50×10^6	1 mL	48 well	1.13 cm
5×10^6	2 mL	24 well	1.60 cm
10×10^6	4 mL	12 well	2.26 cm
25×10^6	10 mL	6 well	3.50 cm
Total cell number	**Medium volume to add**	**Culture dish**	**Dish diameter**
2.25×10^7	90 mL	small	3.5 cm
5×10^7	20 mL	medium	6 cm
12.5×10^7	50 mL	large	10 cm
25×10^7	100 mL	extra large	15 cm
Total cell number	**Medium volume to add**	**Culture flask**	**Growth area**
6×10^7	24 mL	50 mL	25 cm^2
20×10^7	80 mL	250 mL	75 cm^2
40×10^7	160 mL	720 mL	162 cm^2
60×10^7	240 mL	900 mL	225 cm^2
Total cell number	**Medium volume to add**	**Culture bag**	**Growth area**
6.25×10^7	25 mL	25 mL	27 cm^2
12.50×10^7	50 mL	50 mL	58 cm^2
25×10^7	100 mL	100 mL	112 cm^2

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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